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Article

Prevalence of Azole-Resistant *Aspergillus fumigatus* is Highly Associated with Azole Fungicide Residues in the Fields

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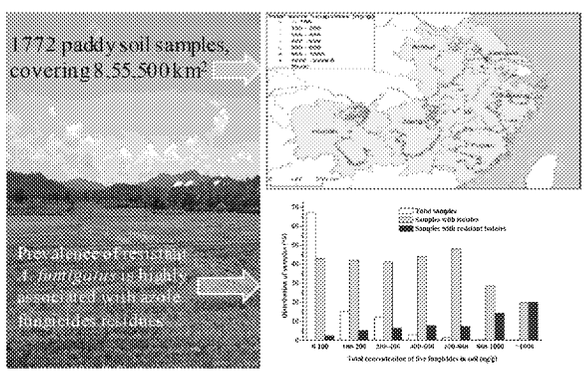
ABSTRACT: Triazole resistance in *Aspergillus fumigatus* is a growing public health concern. In addition to its emergence in the therapy of invasive aspergillosis by triazole medicines, it has been frequently detected in agricultural fields all over the world. Here, we explore the potential link between residues of azole fungicides with similar chemical structure to triazole medicines in soil and the emergence of resistant *A. fumigatus* (RAF) through 855 500 km^2 monitoring survey in Eastern China covering 6 provinces. In total, 67.3%, 15.2%, 12.3%, 2.9%, 1.5%, 0.4%, and 0.3% of the soil samples contained these five fungicides (tebuconazole, difenoconazole, propiconazole, hexaconazole, and prochloraz) of 0−100, 100−200, 200−400, 400−600, 600−800, 800−1000, and >1000 ng/g, respectively. The fractions of samples containing RAF isolates were 2.4%, 5.2%, 6.4%, 7.7%, 7.4%, 14.3%, and 20.0% of the samples with total azole fungicide residues of 0−100, 100−200, 200−400, 400−600, 600−800, 800−1000, and >1000 ng/g, respectively. We find that the prevalence of RAFs is positively ($P < 0.0001$) correlated with residual levels of azole fungicides in soils. Our results suggest that the use of azole fungicides in agriculture should be minimized and the intervals between treatments expanded to reduce the selective pressure toward the development of resistance in *A. fumigatus* in agricultural fields.



KEYWORDS: *Aspergillus fumigatus, azole fungicides, resistance, microsatellite typing*

1. INTRODUCTION

Aspergillus fumigatus is the most common cause of invasive aspergillosis (IA) affecting humans, especially immunocompromised patients. IA is often fatal with mortality rates of 28.5−75% and difficult to treat.[1−4] Medicinal triazoles are the primary therapeutic agents for IA with only three licensed triazoles, that is, itraconazole (ITZ), voriconazole (VOC), and posaconazole (POC).[5,6] However, triazole resistance in *A. fumigatus* has been frequently reported and has become a growing global public health concern.[6,7]

The resistance in *A. fumigatus* is usually caused by alterations in *cyp51A* gene encoding lanosterol 14-α demethylase that is necessary for the biosynthesis of ergosterol, which is an essential component of fungal cell membrane, and overexpression of efflux pumps and *cyp51A*.[8−11] TR34 (the 34-bp tandem repeat)/L98H and TR46/Y121F/T289A are two of the most prevalent mutations that have been reported in both clinical and environmental azole-resistant isolates worldwide, which are thought to be closely related to the use of azole fungicides in the environment.[12] Besides, TR53 in *cyp51A* in *A. fumigatus* which was responsible for ITZ and VOC resistance and have been discovered in both clinical and environmental samples.[13] Other point mutations such as G54, G138, M220, G448, and P216 also can result in triazole-resistance in *A. fumigatus*.[14−17] In addition, the resistance mechanisms of non-*cyp51A* mutation, such as the upregulation of efflux pump genes including ATP-blinding cassette (ABC) transporters and transporters of the major facilitator superfamily (MFS), have been increasingly reported.[18]

The resistance may emerge in the therapy of patients with medicinal triazoles.[16,19,20] Surprisingly, the resistance has been also found in patients who had not received triazoles.[21] Given that medical and agricultural triazoles share similar structures and the same action site in 14-α-demethylase, there is a longstanding hypothesis that the use of triazole fungicides in agriculture for the control of plant fungal pathogen might be linked to increased azole resistance in *A. fumigatus*.[22−25] Triazole fungicide-driven resistance in *A. fumigatus* was observed in liquid medium under laboratory conditions.[26,27] Thus, it is probable that the use of azole fungicides may lead to the emergence and spread of resistant *A. fumigatus* in the

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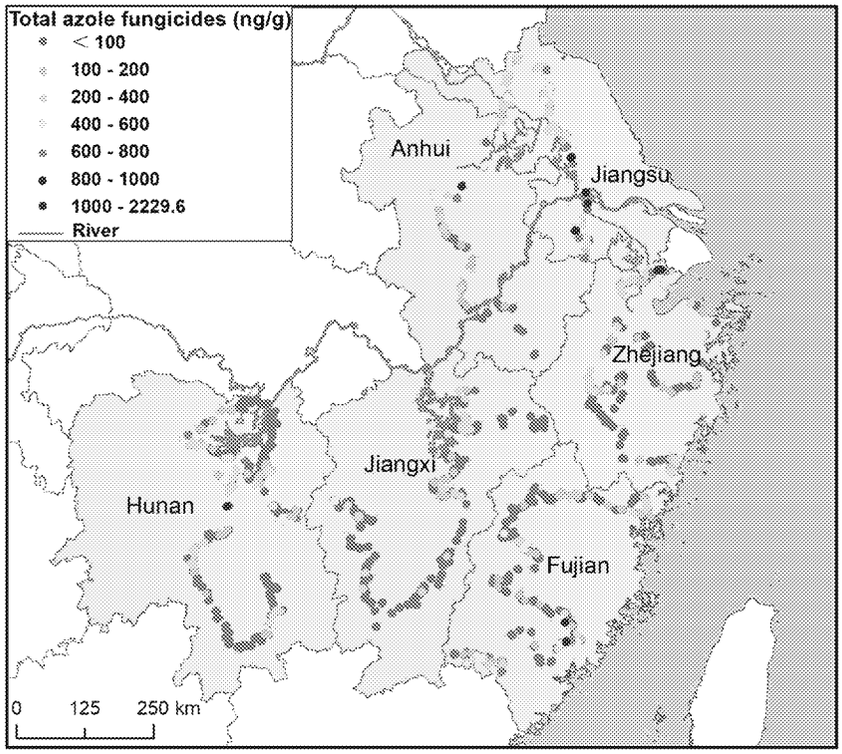

Figure 1. Sample sites and measured total concentrations of the five fungicides in soil.

environment. If proven, this poses a pronounced risk for human health because azole fungicides are heavily used for the control of plant pathogens.[7] In china, the usage of azole fungicides account for more than one-third of all fungicides, amounting to about 27 million kg/year during 2013−2016.[28] However, at present there is little concrete evidence associating the emergence of RAFs to residues of azole fungicides in environmental media such as soil.[25,29] Small-scale studies did not conclusively demonstrate a connection between triazole fungicides and the resistance,[19,29,30] even though the resistance was detected in *A. fumigatus* isolates obtained from azole fungicides treated fields.[31] Here, we attempted to find the possible relationship between residual levels of azole fungicides and the prevalence of RAFs in agricultural soil through an unprecedented, large-scale survey. This study provides solid evidence for the hypothesis that the application of azole fungicides in agriculture drives the emergence and spread of RAF.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials. ITZ (98%), VOC (99.9%), tebuconazole (TBCA) (98.5%), and difenoconazole (DFCA) (98.7%) were purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany. Posaconazole (POC) (99.9%) was purchased from Sigma-Aldrich Co., U.S. Propiconazole (PPCA) (98.3%), hexaconazole (HXCA) (98.4%), and prochloraz (PCRA) (98.3%) were purchased from Beijing Qinchengyixin Technology Development Co., Beijing, China. The enantiomeric purities of R-(−)-tebuconazole and S-(+)-tebuconazole of ≥98.0% were obtained from Daicel Chiral Technologies Co., Ltd., Shanghai, China. Solvents (Methanol, Acetonitrile) used in HPLC separation were all of HPLC grade from Sigma-Aldrich Co., U.S. Primary secondary amine (PSA, 40 μm) was purchased from Agela Technologies (Newark, DE). NaCl, formic acid (FA), acetone and anhydrous $MgSO_4$ of analytical grade were purchased from commercial sources. Sabouraud's dextrose broth medium (SDBM), Potato dextrose agar (PDA) medium, Sabouraud's dextrose agar (SDA), were purchased from Qingdao Haibo Biotechnology Co., China.

2.2. Soil Sample Collection. We randomly collected 1772 paddy soil samples after the harvest of rice grains in an area covering 855 500 km^2. Samples were taken from the top 0−5 cm surface of the fields spanning 6 provinces (Jiangsu-JS, 160; Hunan-HN, 376; Fujian-FJ, 355; Zhejiang-ZJ, 433; Jiangxi-JX, 329; Anhui-AH, 120) (Figure 1) in eastern China from October to November, 2018. At each sampling point, two identical samples were taken for isolation of RAF and analysis of residual azole fungicides. Each sample has a unique number, GPS information, and was sent to the laboratory for storage within 24 h.

2.3. Isolation and Identification of Triazole-Resistant *A. fumigatus* from Soil. For the isolation of *A. fumigatus* from the soil samples, 15 grams of each soil sample were suspended in 80 mL of aseptic 0.85% NaCl solution and shaken on a rotatory shaker at 150 rpm and 30 °C for 2h. Then 100 μL of supernatant was spread onto an SDA plate supplemented with 100 mg/L of chloramphenicol (Sangon Biotech Co., Shanghai, China). Each sample was distributed onto three plates, sealed and placed in an incubator at 37 °C for 72 h. Up to five colonies for each plate were selected. The identification of *A. fumigatus* was performed by their microscopic and morphologic characteristics and sequence analysis of ITS and β-tubulin gene.[28] In order to detect azole-resistant isolates, the MICs of each isolate of *A. fumigatus* against triazole drugs (VOC, ITZ and POC) were determined with the method in Clinical Laboratory Standards Institute document M38-A2.[32] The clinical breakpoints for *A. fumigatus*: MIC > 2 mg/L for ITZ and VOC, and MIC >

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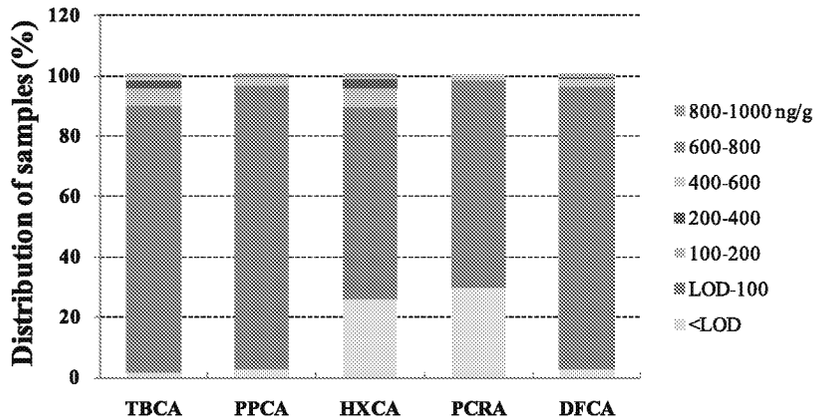

Figure 2. Distribution of measured concentrations of tebuconazole-TBCA, propiconazole-PPCA, hexaconazole-HXCA, prochloraz-PCRA, difenoconazole-DFCA in total soil samples (The values in the legend represent the percentage of measured concentrations of each pesticide in total soil samples).

0.25 mg/L for POC, respectively, was considered "azole-resistant".[32]

2.4. *cyp51A* Gene Sequence of *A. fumigatus*. The genomic DNA was extracted using the Column Fungi Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China). Both of the *cyp51A* and promoter region were amplified by the primers of A7 (5′TCATATGTTGCTCAGC-GG3′) and P450-A2 (5′CTGTCTCACTTGGATGTG3′).[27] The PCR amplification was executed in a 30 μL reaction system, containing 15 μL of 2× PrimerSTAR Max DNA Premix (TaKaRa, Kyoto, Japan), 1 μL of template DNA (approximately 50 ng/μL), 1.2 μL of each primer (10 μM), and 11.6 μL of ddH_2O, at 95 °C for 4 min followed by 38 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final step of 72 °C for 10 min. The sequence was compared to those of an azole-susceptible strain (GenBank accession no. AF338659).

2.5. Analysis of Microsatellite Type. Genotyping was performed on all resistant isolates of *A. fumigatus* by measuring the repeat numbers of nine microsatellite loci, according to the instruction described previously.[33] Briefly, the short tandem repeat sequences of nine markers (STRAf2A, −2B, −2C, −3A, −3B, −3C, −4A, −4B, −4C) were amplified using nine pairs of primers. The amplification product was determined by addition of the GeneScanTM-500 LIZ Size Standard and subsequent analysis of the fragments on the Applied Biosystems ABI 3700xL Genetic Analyzer. Assignment of repeat numbers for each of the nine microsatellite loci was determined by using the GeneMapper package version 2.0 software (Applied Biosystems). For phylogenetic analysis, a total of 99 RAF including 79 RAF collected in this study and 20 RAF from other countries was used.[32,34−38] The dendrogram is based on a categorical analysis of STR repeat numbers in combination with UPGMA clustering using BioNumerics v7.5 software (Applied Maths, Belgium).

2.6. Azole Fungicides Detection. Soil samples were prepared for examining the following azole fungicides: tebuconazole, propiconazole, hexaconazole, prochloraz, difenoconazole. Extraction of azole fungicides from soil samples was performed by QuEChERS method according to Cui et al.[39] with some modifications. Briefly, each soil sample (5 g, dry equivalent) was weighed into a 50 mL polypropylene centrifuge tube containing 10 mL of milli-Q water and 10 mL of acetonitrile and then shaken vigorously for 10 min using a shaker, immediately followed by ultrasonic extraction for 20 min. The mixture was vigorously vortex-mixed with 4 g of anhydrous $MgSO_4$ and 1 g of NaCl for 1 min and then centrifuged for 5 min at 6000 rpm. Then, the obtained upper phase (1.5 mL) was collected in a 2 mL-centrifuge tube containing 150 mg of anhydrous $MgSO_4$ and 50 mg of PSA. After vortexing for 1 min, the tube was centrifuged for 2 min at 5000 rpm. The supernatant was filtered through a 0.22 μm Nylon syringe filter for UPLC-MS/MS analysis.

2.7. UPLC-MS/MS Analysis. The separation of azole fungicides (tebuconazole, propiconazole, hexaconazole, prochloraz, difenoconazole) was performed using Agilent 1290 liquid chromatography system. The system included a G4220A binary pump, a G4226A autosampler and a G1330B column oven installed with analytical column (Eclipse Plus C18 RRHD, 100 × 2.1 mm i.d., 1.8 um particle size, Agilent Technologies, Santa Clara, CA) at temperature of 40 °C and flow rate of 0.3 mL/min. Mobile phase A was H_2O+0.1% FA and mobile phase B was CH_3OH. The following gradient program was used: 0−1 min, 5% of B; 1−4 min, 5−60% of B; 4−14 min, 60−100% of B. The injection volume was 2 μL. Mass spectrometric analyses were performed by an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies) using the multiple reaction monitoring (MRM) and positive electrospray ionization (ESI+) mode. The concrete MS-MS parameter for these compounds is listed in Supporting Information (SI) Table S1. Agilent Mass Hunter software (version B.04.01, Agilent) was used for data acquisition and processing.

2.8. Quantification and Validation. Five azole fungicides were quantified by external standard method using calibration solutions prepared in blank matrix extract at a series of concentrations (0.0001, 1, 10, 100, 200 μg/kg). The analytical method was verified through systematical evaluation of limit of detection (LOD), limit of quantification (LOQ), recovery, matrix effects, and intra- and interday precision. The assay validation results are presented in SI Tables S2−S4.

3. RESULTS

3.1. Residual Azole Fungicides in Agricultural soils. The residual levels of four triazole fungicides and one benzimidazole fungicide, that is, TBCA, PPCA, HXCA, PCRA, and DFCA, were measured in the retrieved soil samples. These fungicides are extensively used in China, and

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have been previously shown to induce resistance in *A. fumigatus*.[27,40] Overall, TBCA (98.4%) was the most frequently detected in the soil samples, followed by PPCA (97.5%), DFCA (97.2%), HXCA (74.0%), and PCRA (70.5%) (Figures 1 and 2). This indicates that applications of azole fungicides have resulted in their ubiquitous and persistent contamination in agricultural soils, usually as mixtures. The predominant concentration in the soil samples was LOD ~100 ng/g for each of these target fungicides. For HXCA and PCRA, their concentrations in 26.0 and 29.5% of the samples were below the detection limits. In total, 67.3%, 15.2%, and 12.3% of the soil samples contained these five fungicides of 0−100, 100−200, and 200−400 ng/g (Figure 1 and 3), respectively. A

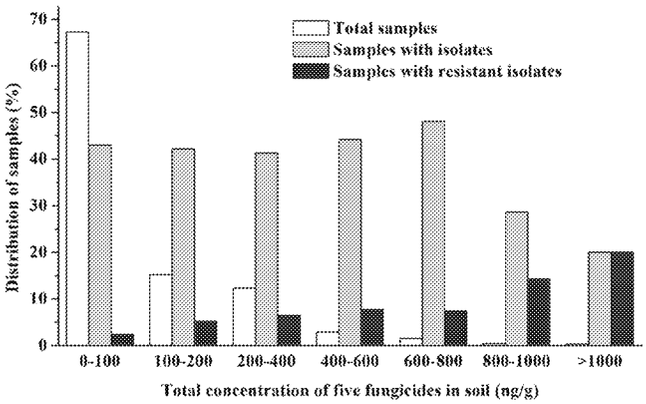

Figure 3. Distribution of total samples (1772 soil samples collected from six provinces), samples with *A. fumigatus* isolates and resistant isolates.

small portion (5.1%) of the samples contained the fungicides at levels exceeding 400 ng/g, including 2.9%, 1.5%, 0.4%, and 0.3% of the samples with azole residues of 400−600, 600−800, 800−1000, and >1000 ng/g, respectively. Regional differences in residual levels of total azole fungicides in soils were observed (Figure 4). The highest levels were found in soils from JS and lowest from HN.

3.2. Azole-Resistant *A. fumigatus* in Soil Samples. Isolation of *A. fumigatus* was performed on all samples. *A. fumigatus* was isolated from 43.1% of the soil samples with fungicides lower than 100 ng/g, and 42.2%, 41.3%, 44.2%,

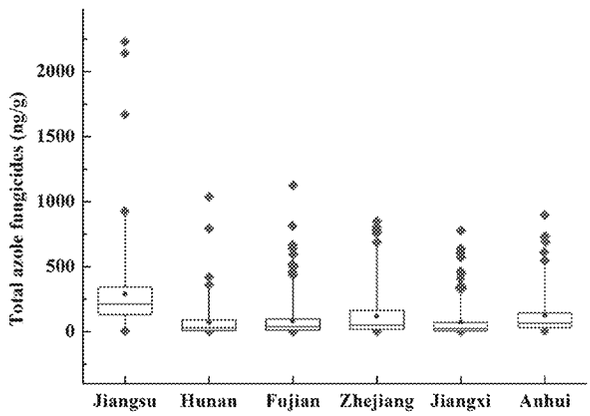

Figure 4. Median and range of total concentrations of five fungicides in paddy soil samples from Jiangsu, Hunan, Fujian, Zhejiang, Jiangxi, and Anhui.

48.1%, 28.6%, and 20.0% of those samples containing fungicides at 100−200, 200−400, 400−600, 600−800, 800−1000, and >1000 ng/g (Figure 3), respectively. The isolation of *A. fumigatus* in the fungicide contaminated soils suggests their widespread presence in paddy fields, and that the occurrence does not seem to depend closely on the levels of fungicide residues in the soil. A total of 1520 isolates of *A. fumigatus* were obtained from 756 out of the 1772 soil samples (SI Table S5−S10). Out of these isolates, 79 isolates displayed triazole resistance (SI Table S5−S10, Table S11), including 35, 16, 16, 7, 3, 1, and 1 RAF isolates from the soil samples with azole residues of 0−100, 100−200, 200−400, 400−600, 600−800, 800−1000, and >1000 ng/g, respectively. The fractions of samples containing RAF isolates were 2.4%, 5.1%, 6.4%, 7.7%, 7.4%, 14.3%, and 20.0% of the samples with total azole fungicide residues of 0−100, 100−200, 200−400, 400−600, 600−800, 800−1000, and >1000 ng/g (Figure 3), respectively. The correlation between residual fungicide level and the prevalence of RAF in the soil samples (SI Table S12) was analyzed by Poisson regression. SI Table S13 showed the fit results of Poisson regression. The regression equation between residual fungicide level (x) and prevalence of RAF in the soil (y) is listed as follows: $y = e^{-3.5316+0.0016x}$. The results indicated that the occurrence risk of RAF in the soil samples increased with the increasing azole fungicides residues ($P < 0.0001$). Redundancy analysis (RDA) was used to explore the level of these five azole fungicides affecting occurrence of RAF. The proportion explained of RDA1 and RDA2 were 78.6% and 9.4%, respectively. The result revealed that DFCA exhibited the greatest influence on occurrence of RAF and followed by PPCA (SI Figure S1).

3.3. Characterization of Azole-Resistant *A. fumigatus* Isolates. Of 79 RAF strains, 68 were resistant to ITZ with MIC of 8 to >16 mg/L, 67 were resistant to VOC with MIC of 4 to >16 mg/L, and 51 were resistant to POC with MIC of 0.5 to 2 mg/L. To explore the underlying mutations of isolated resistant *A. fumigatus*, the *cyp51A* and promoter regions were amplified and sequenced. There were five different mutations (SI Table S11). The majority of the obtained RAF isolates were found to harbor mutations TR46/Y121F/T289A (35 isolates, 44.3%) and TR34/L98H with (9 isolates, 11.4%) or without S297T/F495I (21 isolates, 26.6%) in *cyp51A*. The other mutations in the resistant isolates were TR53 (2 isolates, 2.5%) and F46Y/G89G/M172V/N248T/D255E/L358L/E427K/C454C (3 isolates, 3.8%). Nine resistant isolates did not harbor any mutation in *cyp51A*. Most isolates (32 out of 35) carrying TR46/Y121F/T289A in *cyp51A* and all isolates with mutations of TR34/L98H and TR53 were found to be resistant to both of ITZ and VOC. The resistant isolates harboring mutations TR34/L98H/S297T/F495I and F46Y/G89G/M172V/N248T/D255E/L358L/E427K/C454C were all resistant to ITZ. The prevalence of POC-resistance was substantially lower than that for ITZ or VOC.

3.4. Microsatellite Type of the Isolated Azole-Resistant *A. fumigatus*. Genotyping results of azole-resistant *A. fumigatus* isolates obtained in this survey showed a large genotypic diversity, not only among strains with different resistance mechanisms (TR46/Y121F/T289A, TR34/L98H, TR34/L98H/S297T/F495I, TR53, no mutation type) but also in those with the same resistance mechanism (Figure 5). Among the strains with TR46/Y121F/T289A, two isolates ZJC-64Y-1 and ZJL-43Y-1 obtained from Zhejiang were genetically indistinguishable. The same situation was also

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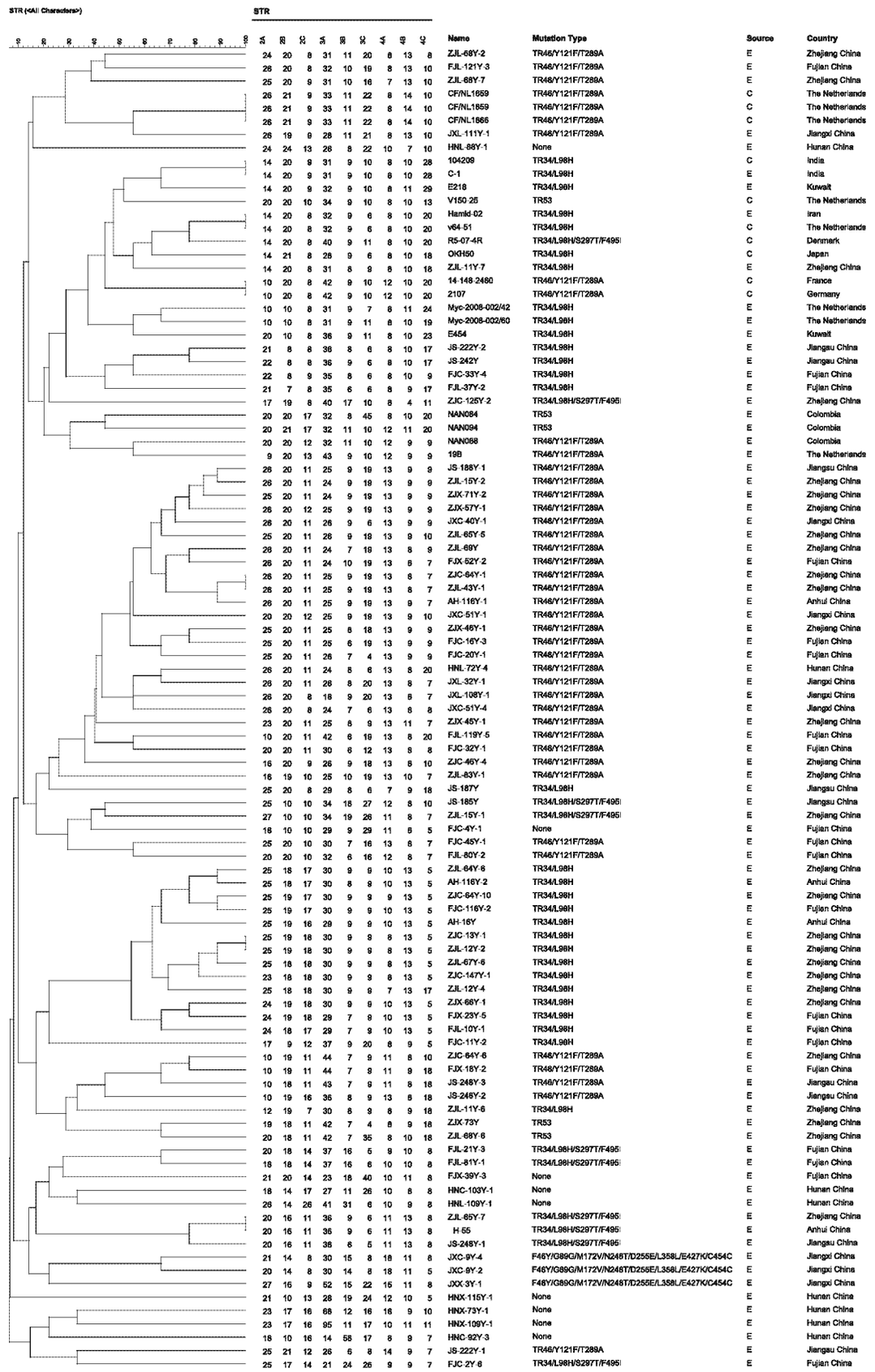

Figure 5. Genotypic relationships between azole-resistant *A. fumigatus* strains from environmental samples in this survey and published isolates from other countries. The scale bar indicated the percentage identity. E, environmental isolate; C, clinical isolate.

found in strains ZJC-13Y-1 and ZJL-12Y-2 with mutation of TR34/L98H. Moreover, strain ZJL-65Y-7 isolated from Zhejiang and the strain AH-55Y from Anhui shared the same mutation and genetic relationship. However, most of the

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resistant strains are genetically distant, even if they are from the same province. A similar genotypic relationship was also observed in resistant strains with other mutation mechanisms, such as TR34/L98H/S297T/F495I, TR34/L98H, TR53. Besides, no genetic similarity was found in azole-resistant strains with *cyp51A* wild type. In general, azole-resistant strains with different resistance mechanisms collected in different provinces have significant geographical and genetic differences.

4. DISCUSSION

Azole resistance in *A. fumigatus* has been widely reported worldwide, which has become an increasing public health issue. With the increased prevalence of resistant *A. fumigatus* in agricultural soil all over the world, fungicide-driven route for development of azole resistance in *A. fumigatus* has be proposed for a decade.[26,27,29,41] However, there is little evidence obtained from realistic fields.

In our study, the frequency of RAF isolates in soils was 8.6% (65/756) based on *A. fumigatus*-positive samples, which similar to the range of environment azole-resistance rates reported in other regions in the world (SI Table S14). Although, it is significantly higher than those of 1.6% and 2.1% reported from China by Chen et al.[42] and Ren et al.,[27] respectively. it is similar to the isolate rate of 7.9% more recently found by Chen et al.[28] Profoundly, the proportion of the samples containing isolated RAFs is highly ($P < 0.0001$) associated with the level of azole fungicides (Figure 3, SI Table S12–S13). Moreover, regional differences in the prevalence of RAF isolates (SI Table S15) also indicated that highest isolation frequency (11.3%) occurred in JS, coinciding with its highest levels of fungicide residues, and lowest (4.9%) in HN, corresponding to its lowest levels of azole fungicide residues. Our previous study showed that high-concentration is more likely induce triazole resistance in *A. fumigatus* under laboratory-simulated conditions.[39] Therefore, these findings together suggest that the prevalence of RAFs in agricultural soils is closely related to residual levels of azole fungicides, attributable to their widespread use in protecting plants. Just like triazoles drugs used in medical treatment, triazole fungicides inhibit 14α-demethylase. These fungicides are used worldwide in agriculture for the control of plant fungal pathogens, and they are applied by spraying onto plants or coating seeds.[43−45] Given that most of the soil samples contain at least four azole fungicides (Figure 2), most of the fields received successive applications of individual or mixtures of these pesticides. Repeated and sustained applications of azole fungicides for plant protection may lead to an increased selective pressure for resistance development in *A. fumigatus*. The residues in agricultural soils resulting from the widespread application of these agricultural fungicides drive the development of triazole resistance in *A. fumigatus*. This was in agreement with the fact that the evolution from sensitive to resistant *A. fumigatus* was observed after successive exposure to triazoles.[26,27] This may explain why no resistance was detected in *A. fumigatus* collected from soil treated by spraying the triazole fungicide tetraconazole only twice a year for more than 15 years.[30] This suggests that agricultural fields receiving repeated azole fungicide applications are likely the reservoirs of RAFs. Thus, extensive use of azole fungicides may have lasting risk for human health care since RAFs, induced by residual triazole fungicides in soils, may be transported by dispersal in air[46] or the food chain.[7]

Mutations in *cyp51A* with or without TR insertion in its promoter are the primary resistance mechanism.[47] The mutations TR46/Y121F/T289A, TR34/L98H, and TR53 were previously reported to confer triazole resistance in *A. fumigatus*[48−52] and are frequently detected in environmental and clinical RAF isolates (SI Table S16). In this study, we found that these three mutations account for 84.8% of all resistance mechanisms (67/79). These observations indicated that fungicide residues in agricultural fields lead to a variety of genetic changes and thus facilitated the evolution of resistance in *A. fumigatus*.

The microsatellite typing analysis of RAF isolates in this survey showed the information about the emergence of azole resistance in China differed from those found in other countries. Diverse genotypes are included in the present RAF strains. Most of these RAFs were genetically unrelated with each other, which suggested that these resistant strains may develop de novo rather than from soil migration. Besides, there is increasing evidence that sexual cycle of *A. fumigatus* also plays an important role in its resistance development. Camps et al. found that TR34/L98H strains could outcross with wild type isolates with diverse genetic backgrounds.[53] The sexual crossing experiments conducted by Zhang et al. found a new mutation of TR46^3/Y121F/T289A.[54] Different from asexual reproduction, sexual reproduction produces new genotypes. Considering the diverse genotypes among RAF isolates in this study, sexual reproduction may be participated in the emergence and spread of RAF.

Environmental Implications. Azole compounds have been widely used for controlling crop diseases in agriculture due to their high-efficiency and broad-spectrum. *A. fumigatus* shares the natural environment with plant pathogens and are also exposed to selective pressure from azole fungicides. The present study comprehensively assessed the impact of azole fungicides on the prevalence of azole-resistant *A. fumigatus* in field soils. The emergence of resistant *A. fumigatus* in fields was found to be closely ($P < 0.0001$) related to residual levels of azole fungicides in this large-scale survey. The obtained results provide critical evidence linking prevalence of resistant *A. fumigatus* in agricultural fields to the applications of azole fungicides. The usage of azole fungicides in agriculture is far higher than that of medical azoles,[55] which will inevitably lead to the emergence and spread of resistant *A. fumigatus*. The resistance conidia could transport via dispersal in air[46] or the food chain,[7] and finally be inhaled by immunocompromised patients, leading to human resistance to triazole medicines and thus increased numbers of treatment failures. At present, there is no effective alternative for azole fungicides in plant protection.[56] To reduce the selective pressure for the development of the resistance in *A. fumigatus*, it is suggested that azole fungicides should be applied as sparingly as possible, and that the interval between applications be as long as possible.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c03958.

Tables S1−S4 showing the information on the MS/MS parameters, linearity and ME of azoles fungicides in diverse soils, recovery experiments with LOD, LOQ, intraday and interday variability. Tables S5−S10

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showing the coordinates of soil samples, measured concentrations of five azole fungicides, and isolation of AF and RAF from JS, HN, FJ, ZJ, JX, and AH provinces (XLS)

Tables S11−S16 showing the total RAF isolates from soil samples, their MIC against ITZ, VOC and POC, and detected mutations in *cyp51A*, distribution of residual mean concentration of five azole fungicides and number of samples and samples with RAF, Results summary of Poisson regression, isolation rate of RAF in different countries, regional difference in the prevalence of total and resistant *A. fumigatus* isolates, and global distribution of clinical and environmental RAF isolates with different mutations (XLSX)

Figure S1 showing the RDA analysis of occurrence of AF and RAF in association with five azole fungicides (PDF)

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